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Evolution

Skeletal matrices, muci, and the origin of invertebrate calcification

(Proterozoic–Cambrian transition/bivalves/corals/calcium carbonate skeletons/immunology)

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ABSTRACT **The sudden appearance of calcified skeletons among many different invertebrate taxa at the Precambrian–Cambrian transition may have required minor reorganization of preexisting secretory functions. In particular, features of the skeletal organic matrix responsible for regulating crystal growth by inhibition may be derived from mucous epithelial excretions. The latter would have prevented spontaneous calcium carbonate overcrusting of soft tissues exposed to the highly supersaturated Late Proterozoic ocean [Knoll, A. H., Fairchild, I. J. & Swett, K. (1993) *Palaios* 8, 512–525], a putative function for which we propose the term "anticalcification." We tested this hypothesis by comparing the serological properties of skeletal water-soluble matrices and mucous excretions of three invertebrates—the scleractinian coral *Galaxea fascicularis* and the bivalve molluscs *Mytilus edulis* and *Mercenaria mercenaria*. Crossreactivities recorded between muci and skeletal water-soluble matrices suggest that these different secretory products have a high degree of homology. Furthermore, freshly extracted muci of *Mytilus* were found to inhibit calcium carbonate precipitation in solution.**

The Proterozoic–Cambrian transition is marked in the sedimentary record by the sudden appearance of mineralized skeletons, in particular of calcium carbonate. Except for the calcified *Cloudina* and associated fossils of the upper Vendian, calcification mechanisms were acquired nearly simultaneously by most invertebrate taxa (1), between 545 and 535 million years ago. Possible causes include an increase in atmospheric oxygen (a prerequisite for the biosynthesis of collagen) (2–4), biomechanical requirements (5), increasing calcium levels in ocean water (6, 7), and the appearance of active predation and grazing in the food chain (8).

In addition to any extrinsic trigger, the fact that highly organized skeletons appeared nearly simultaneously in many different taxa requires an intrinsic mechanism. Some authors believe that the introduction of the calcifying machinery represents a major reorganization of calcium metabolism within the cell (9). In this paper we explore the hypothesis that for the implementation of calcification, only modest alterations were required of the preexisting repertoire of excretory functions within the Metazoa. Our hypothesis predicts that in different metazoan taxa the individual excretory products contained in calcified and noncalcified tissues are, at least in part, genetically related. Testing the hypothesis therefore includes a comprehensive structural and functional comparison, preferably in a representative selection of the skeleton-bearing metazoan taxa. This study represents the first step in this procedure. Three metazoan systems were investigated—the scleractinian coral *Galaxea fascicularis* and the bivalve molluscs *Mytilus edulis* (edible mussel) and *Mercenaria mercenaria* (American clam). In these organisms, mucus excreted by the uncalcified outer epithelial skin was serologically compared with water-soluble organic macromolecules extracted from the exoskeletons (soluble matrix, SM). In addition, the capacity of these materials to inhibit the *in vitro* precipitation of $CaCO_3$ was investigated.

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MATERIALS AND METHODS

Living specimens of *Mytilus edulis* and *Mercenaria mercenaria*, supplied by a wholesale firm in Scheveningen, the Netherlands, were cleaned by removing contaminating materials from the shell and subsequently immersed in synthetic seawater (Instant Ocean; Aquarium System). Living colonies of *Galaxea fascicularis* were collected in Okinawa Jima, Japan.

Mucus Extraction. Mucus was extracted by injecting $CaCO_3$ powder (Sigma) through the inhalant siphon on the mussels and clams. Small pellets of mucus were rapidly released. Clean pellets were pipetted and stored at −20°C with 0.1% (wt/vol) NaN_3. The release of coral mucus was obtained by dripping filtered (0.45 μm) natural seawater on the colony surface. The mucus was pipetted and stored with 0.1% NaN_3.

Mucus Purification. After centrifugation of the bivalve muci (2800 × *g*, 30 min), the supernatant was filtered (Millipore; 0.45 μm) and desalted (Amicon; cutoff, 1 kDa). The centrifugation residue was suspended in water, decalcified in diluted acetic acid (pH 4), and recentrifuged (2800 × *g*, 30 min). The new supernatant was treated as the first one. The two supernatants were lyophilized.

The filtered seawater containing the soluble coral mucus was centrifuged (3500 × *g*, 30 min), and the supernatant was dialyzed (Sigma dialysis bag; cutoff, 12 kDa) against distilled water with 0.1% NaN_3 and concentrated by ultrafiltration (cutoff, 10 kDa). The solution was dialyzed against distilled water and lyophilized.

Extraction of SM. The cleaned bivalve shell powders were suspended in water and decalcified by dripping acetic acid up to pH 4. The decalcification, controlled by a titrimeter, was completed within 16 hr. After centrifugation (2800 × *g*, 20 min), the supernatant containing the acid-soluble matrix was filtered (Millipore; 1.2 μm), desalted (Amicon; 1 kDa) and lyophilized.

To remove animal tissues and algal contaminants, the coral skeletons were boiled successively in 0.3 M NaOH, water, and 1% (vol/vol) NaOCl solution and then rinsed and crushed. The powder was decalcified with 10% (wt/vol) EDTA (pH 8) in 0.1% NaN_3. The solution was dialyzed three times against distilled water (Sigma, dialysis bag; cutoff, 12 kDa), concentrated by ultrafiltration (10 kDa), centrifuged (70,000 × *g*, 1 hr) and lyophilized.

Antibody Production. Polyclonal antibodies were produced in White rabbits from aliquots of the lyophilisates (Eurogentec, Seraing, Belgium). Following the first injection (t_0), three immunizations were performed after 14, 28, and 56 days. The

Abbreviations: DTE, dithioerythritol; SM, soluble matrix.

bleedings were made before t_0 (preimmune serum) and 38 and 66 days after t_0. The antiserum against the SM of *Mercenaria mercenaria* was raised previously (10).

Enzyme-Linked Immunosorbent Assay (ELISA). The immunological reactivities were quantified by spectrophotometric ELISA (11) on Nunc Maxisorb microplates. The protocol, described elsewhere (12), used alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma; A 8025) as second antibody.

To show the specificity of the antisera against the muci and SMs of *Mercenaria mercenaria* and *Mytilus edulis*, negative controls were done using the soft tissues of these organisms. Lack of material precluded the execution of this test with the anti-*Galaxea* antibodies. Cleaned inner fragments of adductor muscles of the two bivalves were crushed under liquid nitrogen. A soluble extract was produced and its reactivity with anti-mucus and anti-SM antisera was compared with that obtained from the same amounts of mucus and SM. As the reactivities with the muscle extracts were very low, we concluded that the anti-*Mytilus* and anti-*Mercenaria* sera did not contain antibodies directed against contaminating cellular debris.

The muci and SMs were compared directly by ELISA and, more specifically, with anti-mucus antisera previously adsorbed on EDTA-etched skeletal powders (12) of the two bivalves. The specificity of the adsorptions was controlled by adding to the antiserum another antiserum which did not react to any of the molluscan antigens and by testing the mixture before and after the adsorptions against the target antigens of the latter serum.

Characterization of Antigenic Determinants. To investigate the protein content of the determinants, identical quantities of the antigen preparations were digested with proteinase K (weight ratio 1:50), while others were denatured with SDS buffer [50 mM Tris/4% (wt/vol) SDS/1.6% (wt/vol) dithioerythritol (DTE), pH 6.8], or digested with proteinase K after a short SDS denaturation (3 min, 95°C). The sugar content was studied by oxidation with a 0.05 M sodium periodate (13). All the antigens were incubated for 16 hr at 37°C in these various solutions before being tested by ELISA.

Immunohistological Staining. The distribution of mucus and of SM antigens within a shell radial section of *Mercenaria* was observed with immunohistology according to a published procedure (14). The samples were observed with a binocular microscope under incident light.

***In Vitro* Inhibition of $CaCO_3$ Precipitation.** The addition of HCO_3^- ions to a solution containing Ca^{2+} ions leads to $CaCO_3$ precipitation and a decrease in pH. These processes are delayed when a crystallization inhibitor is added (15). In this study, the formation of protons in solution was balanced by the addition of OH^- ions to maintain a constant pH. All the experiments were performed in synthetic seawater at 25°C. Purified SMs and muci and freshly extracted unpurified muci of *Mytilus* were tested for their crystallization-inhibiting properties.

RESULTS

ELISA. Comparison of the reactivities of the mucus and the SM with the anti-mucus and the anti-SM antisera revealed remarkably strong reactivities for all the preparations in each of the three species (Fig. 1). Invariably, the reactions between mucus and anti-mucus and those between SM and anti-SM

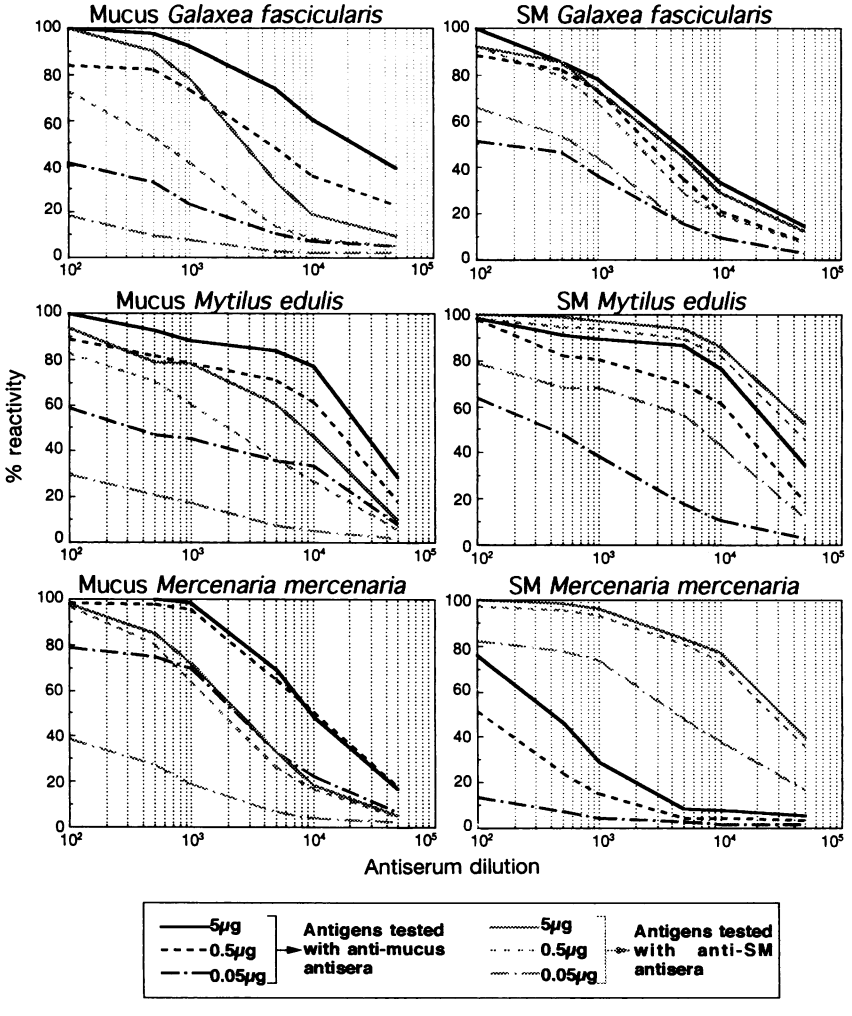

FIG. 1. Crossreactivity ELISAs. Each of the six antigens was tested against its two corresponding antisera: anti-mucus (black lines, continuous and broken) and anti-SM (gray lines, continuous and broken). Antigens were tested in the range from 5 µg to 0.5 ng per well. Results are shown here for three antigen amounts: 5 µg, continuous lines; 0.05 µg, dashed lines; 0.05 µg, dash–dot lines. The relative immunological reactivities (%) were calculated from the highest absorbances (100% for antiserum diluted 1:100 against 5 µg of its target antigens). In each case, high crossreactivities were recorded.

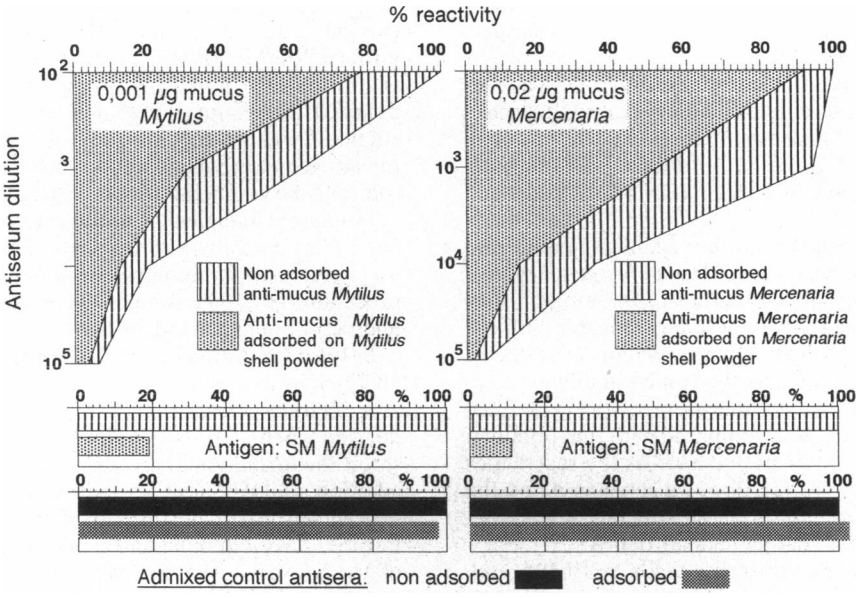

FIG. 2. Reactivity tests with the anti-mucus antisera of *Mytilus* and *Mercenaria* before and after four adsorptions on their respective shell powder. In the two graphs (*Top*), the striped areas represent the percentage of the reactivities removed by adsorption. The efficiency of the adsorptions was controlled on SM antigens (histograms, *Middle*): a complete loss of reactivity was recorded. The specificity of the adsorptions was tested with admixed antisera (anti-polysaccharide of *Emiliana huxleyi* and anti-SM of *Lucina* sp., a heterodont bivalve), which did not crossreact with *Mytilus* and *Mercenaria* antigens (histograms, *Bottom*). When tested on their target antigens (polysaccharide from *Emiliana huxleyi*, SM of *Lucina*), these adsorbed antisera exhibited no decrease in their reactivities.

were a bit stronger than the crossreactivities (i.e., those between mucus and anti-SM and between SM and anti-mucus). These differences were more apparent when low amounts of antigens (0.05 or 0.005 µg) were applied to the ELISAs. The strongest crossreactivities were obtained in *Mytilus* and the weakest ones in *Mercenaria*, leaving *Galaxea* in an intermediate position.

More specific indications of the similarity between muci and SMs were given by the adsorption tests (Fig. 2). For *Mytilus* and *Mercenaria*, the adsorptions of the anti-mucus antibodies on the powders proved to be efficient, as this treatment reduced the reactivities with the SMs by 80% and 90%, respectively. However, when the adsorbed antibodies were tested against the respective muci, up to 30% and 40%, respectively, of the original reactivity had been removed. All the adsorptions were sufficiently specific, as they did not affect the reactivity between an admixed "unreactive" antiserum and its antigen.

These results show that striking similarities exist between the muci and the SMs in each one of the three species. These materials are by no means identical, however. The adsorption experiments suggest that in *Mytilus* and *Mercenaria* about one-third to one-half of the determinants of the muci are also present in the SMs, whereas the other part is unique to the muci.

Characterization of Antigenic Determinants. Proteinase K alone failed to digest any proteinaceous fraction of the muci and the SMs (Fig. 3). However, pretreatment of the samples with SDS/DTE (plus heating) stimulated the enzymatic degradation: a complete collapse of reactivity was recorded in all cases. The SDS/DTE treatment alone provoked an intermediate decrease in reactivity. Sodium periodate treatment had a limited effect on the SM of *Mercenaria* and the mucus of *Mytilus*; it was more effective for the mucus of *Mercenaria* and the SM of *Mytilus* and very effective for both the mucus and the SM of *Galaxea*, where the reactivities were entirely suppressed.

These data suggest that antigenic determinants of the muci and the SMs of *Mercenaria* and *Mytilus* are predominantly proteinaceous (up to 95% of the mucus of *Mytilus*) whereas the integrity of those of *Galaxea* would depend on a combination of protein and polysaccharide moieties.

Immunohistology. Fig. 4 represents immunohistological staining of the outer and median layers of *Mercenaria mercenaria*. The staining with the anti-mucus antibody (Fig. 4*B*) was less intense than that resulting from the incubation with anti-SM (Fig. 4*C*). The two stainings were specific, since the preimmune serum did not stain the sample (negative control, Fig. 4*A*).

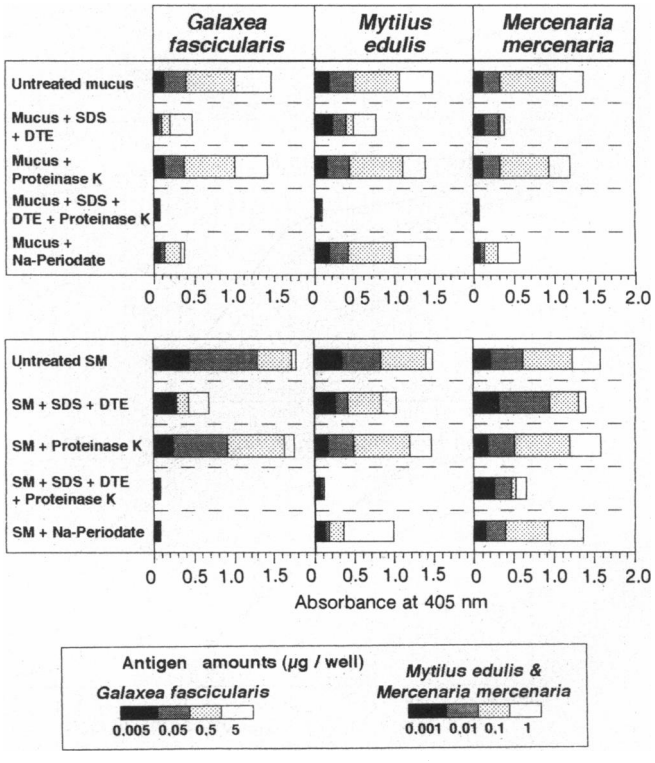

FIG. 3. Chemical nature (proteinaceous or polysaccharidic) of antigenic determinants of mucus and SM of the three studied species. Samples denatured with 4% SDS/1.6% DTE were boiled 1 min. For more detail, see text.

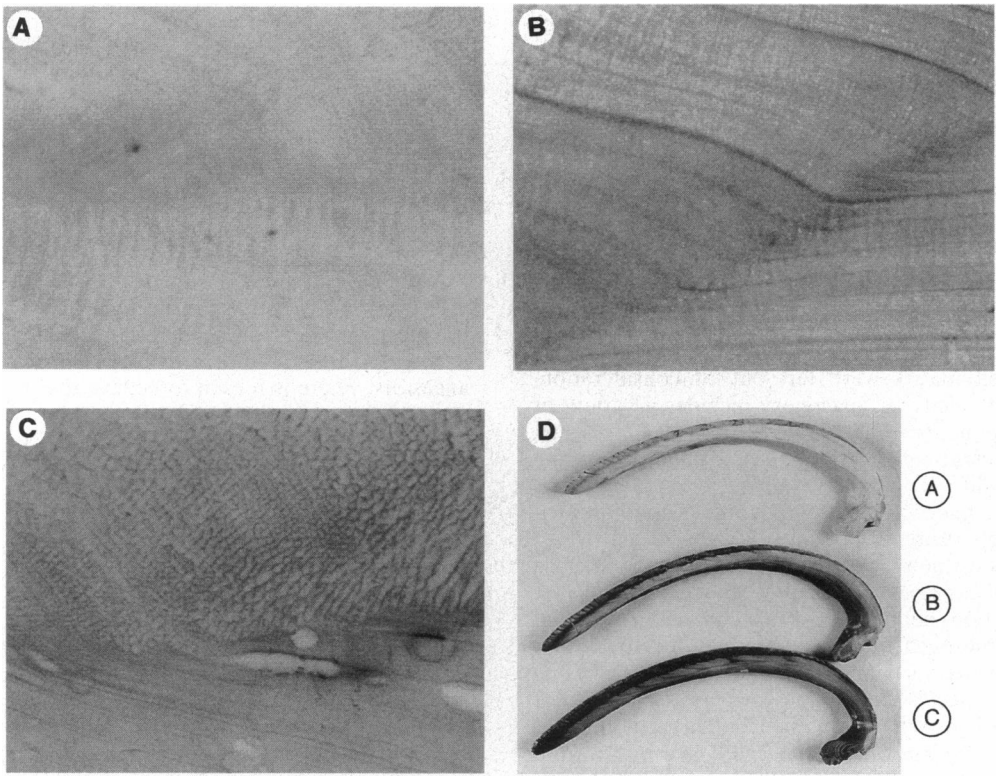

FIG. 4. Immunohistological staining of radial sections of the three-layered shell of *Mercenaria mercenaria*. *A–C* represent the transition between outer (composite prismatic, top) and median (crossed-lamellar, bottom) layers. (*A*) Preimmune serum (negative control), dilution 1:400. (*B*) Anti-*Mercenaria* mucus, dilution 1:400. (*C*) Anti-*Mercenaria* SM, dilution 1:1000. (×70.) (*D*) Macrophotography of the stained samples. (×0.9.)

Although ELISAs detect a high degree of similarity between the mucus and the SM of *Mercenaria*, the target antigens of the respective antisera do not have the same localization within the shell. In the outer composite prismatic layer, the anti-SM antibodies were concentrated both on the organic sheaths surrounding the prisms and on growth planes which run more or less parallel to the inner shell surface, as reported previously (14). In that same layer, the anti-mucus antibodies were found only on the growth planes, whereas the prism outlines were only very faintly stained. In the transition zone and the crossed-lamellar layer, the two antibodies had the same effect: only the growth planes were stained, and the banding was continuous with that in the composite prismatic layer.

These observations indicate that the determinants which the SM and the mucus have in common are predominantly concentrated on the growth surfaces, whereas those determinants of the SM which are absent in the mucus are situated in the sheaths that surround the prisms.

***In Vitro* Inhibition.** The SM of *Mytilus* inhibited the precipitation of aragonite when administered in concentrations as low as 5 μg in 40-ml solutions (Fig. 5). A strong inhibiting capacity was also recorded with the SM of *Galaxea*. In our hands the capability to inhibit crystallization was much less for the SM of *Mercenaria*: this may be related to the low calcium binding affinities of this SM in comparison to other molluscan SMs (16, 17). Alternatively, this function may have been lost by degradation of the organic material while it was contained in the shell, or by our own manipulation.

Also the purified muci did not inhibit crystallization, even when the concentrations were increased up to 50–100 μg per 40 ml. But all experiments performed with a freshly extracted and untreated preparation of *Mytilus* mucus proved positive; a high level of inhibition was recorded. The discrepancy between results obtained with fresh and purified muci may also be explained by an extremely rapid degradation of the inhibitors or by their removal during mucus purification.

These experiments suggest that the SMs as well as the fresh muci can inhibit the crystallization of aragonite. This faculty is labile and easily lost, however. Further study of this phenomenon is required before definitive conclusions can be drawn: this concerns particularly the chemical nature of the mucus inhibitors.

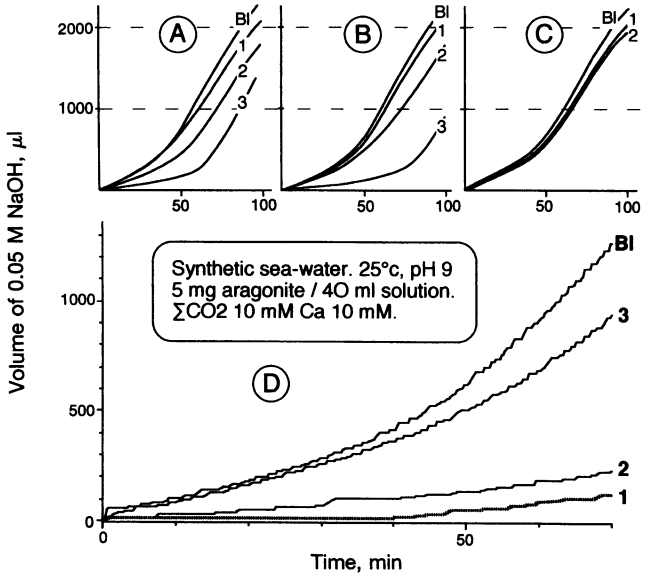

FIG. 5. *In vitro* inhibition of $CaCO_3$ crystallization. (*A*) *Galaxea fascicularis*. Curves: Bl, blank; 1, mucus, 100 μg; 2, SM, 25 μg; 3, SM, 50 μg. (*B*) *Mytilus edulis*. Curves: 1, mucus, 70 μg; 2, SM, 5 μg; 3, SM, 20 μg. (*C*) *Mercenaria mercenaria*. Curves: 1, mucus, 30 μg; 2, SM, 30 μg. (*D*) *Mytilus edulis* (detailed unsmoothed curves.) Curve 1, unpurified mucus, freshly extracted, 800 μl (≈100 μg); 2, SM, 20 μg; 3, SM, 5 μg.

DISCUSSION

Partial Homology of SMs and Muci. The evidence presented in this paper consistently indicates that a partial homology exists between the SMs and the muci in the three investigated metazoans. This result supports our hypothesis that the calcifying machinery of the Metazoa is derived, at least in part, from noncalcifying precursors of the excretory system.

Some objections may be raised against this conclusion, however. First, the three species to which the serological comparison was restricted are not representative of all the Metazoa. Second, only limited fractions of the calcified matrix and the noncalcified excretory products were compared. For example, the leathery periostracum and the less soluble fractions of the shell matrix were left out of consideration. Furthermore, the calcifying machinery includes a variety of nonskeletal components which were not studied here—e.g., pumping devices maintaining supersaturated conditions in the extraepithelial fluids. It remains to be seen whether homologues of all these materials are components of the noncalcifying secretory repertoire, as our hypothesis predicts.

The limitations of the serological technique further reduce the significance of our result. We cannot exclude the possibility that the crossreactions between the SMs and the muci resulted from fortuitous nonspecific interactions, even if no reactions were obtained in the various negative controls. A sobering reminder in this respect is the example of an antiserum against β-fructosidase crossreacting with many plant proteins and hemocyanin of *Helix pomatia*, where a small glycan proved to be the common epitope (18). Finally, the mucus-related component of the SMs may not be a functional part of the calcifying machinery, as it may be a contaminant enclosed in the growing skeleton by entrapment from the extraepithelial calcifying fluid.

There is evidence contradicting this latter possibility, however. First, if contaminating mucus were entrapped in the shell, one would expect to find there all the mucus determinants and not only a selection of them, as was borne out by our adsorption experiments. Second, the SM is intracrystalline and therefore intimately associated with the mineral phase (19). The pretreatment with NaOCl destroyed all the intercrystalline matrix and contaminants. Finally, there is ample evidence to suggest that, especially in the actively growing part of molluscan shells, the organic material at the growth surface plays an important role in calcification and is not passively adsorbed by the mineral. This is well documented, for instance, in the nacreous layer in the cephalopod mollusc *Nautilus pompilius* (20, 21).

Different Functions of Mucus and SM. In view of the suspected partial homology between uncalcified muci and SMs, a close functional relationship should be particularly apparent between these two excretory products. At first sight there seems to be little evidence in support of this supposition.

Metazoan mucous substances serve a remarkable range of different functions. They may act as lubricants, play a crucial role in the entrapment and transport of food particles or in the rejection of deleterious debris (22), are an essential component of the locomotory system of the gastropods, and may provide protection against desiccation (23). In molluscs, they are even considered to act as a primitive immune system (24).

The function of the organic matrix is to keep in check the crystal growth, a process driven by the supersaturation of the fluid medium. This regulatory role has been a subject of intense research (19, 25, 26). In the molluscs, the periostracum around the shell acts as a physical support on the inside of which crystallization is controlled. The crystals and the matrix are segregated from an "extrapallial fluid" excreted by the outer mantle epithelium. The polysaccharides and glycoproteins of the polyanionic SM interact directly with the nascent and growing crystallites of the skeleton (19). When free in solution, these substances may act as crystallization inhibitors. When attached to a solid substratum, they may either facilitate crystal nucleation and epitaxial growth or terminate the crystal growth (15). The sheath-like insoluble matrix forms small chambers which are separated from the extrapallial fluid. The walls of these chambers provide the physical support which allows the reactive polyanionic SM first to stimulate and then to block the crystallization process. The available evidence indicates that all metazoan calcifying systems utilize organic matrices with soluble polyanionic inhibitors as their most reactive components.

Anticalcification and Calcification. The assumption that part of the SM is derived from the noncalcified mucus raises the question whether its inhibitory capacity was already functional in the muci of uncalcified Late Precambrian Metazoan ancestors. There is reason to believe that this was indeed the case.

Consistent sedimentological data (27) show that the Late Proterozoic oceans were probably highly supersaturated with respect to $CaCO_3$ in comparison to the moderate degree of supersaturation of the present oceans (28, 29). Soft-bodied Metazoans probably had to develop inhibitory adaptations to prevent spontaneous overcrusting of their tissues by $CaCO_3$ crystals. The muci excreted by their epithelia may have filled this role. We propose the term anticalcification for this protective and inhibitory function of the muci. This anticalcifying function would have made the muci readily available as SMs in the emergent calcifying systems at the Precambrian–Cambrian transition.

With the well-studied molluscs as an example, we may envisage the following hypothetical scenario for the origin of their calcifying system. The noncalcified ancestors are supposed to have been surrounded by a leathery precursor of the periostracum (30, 31). Between this protective sheet and the outer mantle epithelium was a thin fluid compartment, the precursor of the extrapallial fluid, episodically supersaturated with respect to $CaCO_3$. The same mucus was used in its soluble state to prevent spontaneous crystallization in this compartment and on those parts of the mantle directly exposed to the ocean. For calcification to take place, small chambers had to be formed near the inner surface of the periostracum. If supersaturation were maintained in these chambers by diffusion from the extrapallial fluid, and if the mucus were anchored to the chamber walls, then organized calcification would have proceeded. The spatial ordering of the mucus molecules determined the polymorph (aragonite or calcite) as well as the orientation of the crystals. Alternatively, it may be the rate of crystallization which dictated the polymorphic type, with aragonite precipitating more rapidly and calcite more slowly (32).

This hypothetical scenario is corroborated by the following observations: muci have been suspected to play a role in $CaCO_3$ crystal formation among corals (33) and molluscs (34, 35). Furthermore, among chordates, the intestinal mucus of the seawater eel *Anguilla anguilla* L. was shown to induce the precipitation of calcite crystals (36).

If all calcifying systems have a common origin (37), this may explain why some strong "but unexpected" crossreactivities were found between the SMs of unrelated taxa, such as brachiopods, molluscs, and corals (F.M., unpublished data). These findings are not restricted to lower invertebrates: crossreactivities were found independently (38) between the molecules involved in calcification of two different deuterostomes, an echinoderm and an ascidian.

If the mucous substances with their multiple functions can indeed be considered as the precursors of the SMs, their further study may open a new field in biomineralization research.

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